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INTRODUCTION

Many attempts have been made to correlate the appearance of stainable myelin in the nervous system with the onset of function in developing tracts and nerves (Tilney & Casamajor, 1924; Langworthy, 1926, 1928 *a*, *b*, 1930, 1933; Angulo y Gonzalez, 1929). Langworthy (1933) gives a good review of the literature and comes to the conclusion that medullation occurs in different fibre systems in the order of their phylogenetic development, and that normal function and medullation are closely correlated in their time of onset.

The sheep foetus is considered a suitable subject for the study of medullation in the nervous system because, unlike most of the animals which have been investigated, it is born in an advanced state of development and the various stages in the functional growth of the nervous system are well known (Barcroft & Barron, 1936, 1937, 1939, 1942).

MATERIAL AND METHODS

Sheep foetuses ranging in age from 53 to 140 days after insemination have been used (Table 1). With the exception of two of these (53- and 63-day-old foetuses) which are aged on the basis of their crown-rump length and weight, all have been obtained by Caesarean section from sheep whose stage of gestation is

Table 1. *Sheep foetuses*

No.	Age in days	Weight in g.	C.R.L. in mm.
—	53	25·5	80·0
—	63	102	142
299	66	97·2	140
340	78	222·2	180
268	78	229·0	192
281	84	348·2	210
331	96	533	250
309	111	1450	355
682	120	2200	390
689	129	1670	360
98	140	4090	470

accurately known. After fixation by injection with 4 % formaldehyde through an umbilical artery the brains were removed, cut into slices not more than 1 cm. in thickness and mordanted in Weigert's primary mordant (bichromate-fluorchrome) for 14 days at 30° C. The pieces were then thoroughly washed in water, dehydrated through absolute alcohol and embedded in either celloidin

or paraffin. The latter method gives good results, and where relatively thin sections (15 μ) are cut these can be affixed to albumenized slides without difficulty by floating on 70 % alcohol, blotting with cigarette paper and drying on the hot plate. Thin sections are infinitely preferable to thick ones for demonstrating early myelin, as the differentiation of the background without destaining the myelin is much simpler.

Sections were then placed for 1 hr. in Weigert's gliabieze, washed, stained for 4–6 hr. in Kultschitzky's haematoxylin at 37° C., rewashed, rinsed in gliabieze and differentiated by repeated short immersions in 0·25 % potassium permanganate followed by weak sulphurous acid.

With this method it has been found possible to show the finest myelin sheaths and at the same time obtain adequate differentiation of the background.

Serial sections throughout the whole brain and parts of the spinal cord have been cut in all but the 84-day-old foetus where only the spinal cord had been obtained.

OBSERVATIONS

In the following description no attempt has been made to ascertain the degree of medullation of individual tracts at every stage, but only the earliest time at which myelin is seen in each tract.

Table 2 gives a summary of the main observations, showing the earliest age at which myelin is seen. Where a trace (tr.) is marked on the table this means that only a very few fibres have a myelin sheath at this age. A + does not necessarily mean that every fibre is medullated but that the majority have some myelin.

No myelin is present in any part of the nervous system of the 53-day sheep foetus, though by 63 days several elements of the nervous system have begun to acquire myelin sheaths. The most obvious of these is the vestibular nerve whose branches can be traced into the lateral, superior and medial vestibular nuclei. A few fibres pass through the latter to decussate with similar fibres of the opposite side and appear to pass to the contralateral vestibular nuclei. The uncrossed vestibulo-spinal tract contains several medullated fibres, and a small number of homolateral ascending fibres can be traced from the superior vestibular nuclei into the medial longitudinal fasciculus as far as the trochlear nucleus.

Of the other cranial nerves the oculomotor and trochlear contain only a trace of myelin while the motor root of the trigeminal, abducens, facial and hypoglossal contain slightly more—as do the ventral spinal nerve roots in the cervical region. In addition only the reticulo-spinal fibres contain any myelin. These arise both from the pons and medulla as scattered fibres converging on the medial longitudinal fasciculus (Fig. 2 A) where they turn caudally with or without decussation. A well-marked group of these fibres is seen in all the foetuses (Fig. 1 B) lying just caudal and parallel to the fasciculi of the abducens

Table 2. *Showing the main fibre systems which have acquired a myelin sheath in foetuses of various ages*

tr. = a very few fibres with a fine sheath. + = many fibres with a myelin sheath extending for at least part of their length.

Nerve or tract	Age in days								
	53	63	66	78	96	111	120	129	140
Ventral roots	—	+	+	+	+	+	+	+	+
III, IV, VI, VII, VIII (vest.), XII	—	+	+	+	+	+	+	+	+
V (motor)	—	+	+	+	+	+	+	+	+
Medial longitudinal fasciculus	—	+	+	+	+	+	+	+	+
Vestibulo-spinal, uncrossed	—	+	+	+	+	+	+	+	+
Vestibulo-mesencephalic, uncrossed	—	+	+	+	+	+	+	+	+
Crossed, vestibular	—	+	+	+	+	+	+	+	+
Medullary reticulo-spinal	—	+	+	+	+	+	+	+	+
Pontine reticulo-spinal	—	+	+	+	+	+	+	+	+
Ventral column of spinal cord	—	+	+	+	+	+	+	+	+
Dorsal roots	—	—	+	+	+	+	+	+	+
V sensory mesencephalic and descending	—	—	+	+	+	+	+	+	+
IX, X, XI	—	—	+	+	+	+	+	+	+
Dorsal spino-cerebellar	—	—	+	+	+	+	+	+	+
Uncinate fasciculus	—	—	+	+	+	+	+	+	+
Cerebellar vermis	—	—	+	+	+	+	+	+	+
Lateral column, spinal cord	—	—	+	+	+	+	+	+	+
VIII descending and cochlear	—	—	—	+	+	+	+	+	+
Crossed vestibulo-spinal	—	—	—	+	+	+	+	+	+
Crossed vestibulo-mesencephalic	—	—	—	+	+	+	+	+	+
Isthmic reticulo-spinal	—	—	—	+	+	+	+	+	+
Solitary tract	—	—	—	+	+	+	+	+	+
Ventral spino-cerebellar	—	—	—	+	+	+	+	+	+
Brachium conjunctivum	—	—	—	+	+	+	+	+	+
Myelin in flocculus	—	—	—	+	+	+	+	+	+
Trapezoid body	—	—	—	+	+	+	+	+	+
Lateral lemniscus	—	—	—	+	+	+	+	+	+
Striae acousticae	—	—	—	+	+	+	+	+	+
Rubro-spinal	—	—	—	+	+	+	+	+	+
Mesencephalic reticular at level of inferior colliculus	—	—	—	+	+	+	+	+	+
Myelin in inferior colliculus	—	—	—	+	+	+	+	+	+
Posterior commissure	—	—	—	tr.	+	+	+	+	+
Posterior column of spinal cord	—	—	—	+	+	+	+	+	+
Olfactory tract	—	—	—	—	+	+	+	+	+
Optic nerve and tract	—	—	—	—	+	+	+	+	+
Myelin in lateral cerebellar lobes	—	—	—	—	+	+	+	+	+
Brachium pontis	—	—	—	—	+	+	+	+	+
Commissure of the lateral lemniscus	—	—	—	—	+	+	+	+	+
Peduncle superior olive	—	—	—	—	+	+	+	+	+
Intercollicular commissure	—	—	—	—	+	+	+	+	+
Spino-tectal	—	—	—	—	+	+	+	+	+
Tecto-spinal	—	—	—	—	+	+	+	+	+
Central tegmental fasciculus	—	—	—	—	+	+	+	+	+
Mesencephalic reticular at level of superior colliculus	—	—	—	—	+	+	+	+	+
Stratum profundum of superior colliculus	—	—	—	—	—	+	+	+	+
Supramamillary decussation	—	—	—	—	+	+	+	+	+
Habenulo-peduncular	—	—	—	—	tr.	+	+	+	+
Stria habenularis	—	—	—	—	+	+	+	+	+
Stria terminalis	—	—	—	—	+	+	+	+	+
Fimbria	—	—	—	—	+	+	+	+	+
Optic radiation	—	—	—	—	tr.	+	+	+	+
Olfacto-septal fibres	—	—	—	—	+	+	+	+	+

Table 2 (*continued*)

Nerve or tract	Age in days								
	53	63	66	78	96	111	120	129	140
Fasciculus and ansa lenticularis	—	—	—	—	+	+	+	+	+
Fasciculus thalamicus	—	—	—	—	tr.?	+	+	+	+
Dorsal and ventral supraoptic decussation	—	—	—	—	+	+	+	+	+
Medial lemniscus	—	—	—	—	+	+	+	+	+
Fibres in substantia nigra	—	—	—	—	tr.	+	+	+	+
Efferents from subthalamic nucleus	—	—	—	—	+	+	+	+	+
External capsule	—	—	—	—	tr.	+	+	+	+
Sigmoid and coronal gyri	—	—	—	—	tr.	+	+	+	+
Olivo-cerebellar	—	—	—	—	+	+	+	+	+
Ventral and dorsal external arcuate	—	—	—	—	+	+	+	+	+
Brachium of inferior colliculus	—	—	—	—	—	+	+	+	+
Brachium of superior colliculus	—	—	—	—	—	+	+	+	+
Myelin in stratum superficiale of superior colliculus	—	—	—	—	—	tr.	+	+	+
Fibres in central grey of mesencephalon	—	—	—	—	—	+	+	+	+
Mamillary peduncle	—	—	—	—	—	+	+	+	+
Anterior column of fornix	—	—	—	—	—	tr.	+	+	+
Mamillo-thalamic tract	—	—	—	—	—	+	+	+	+
Efferents from anterior thalamic nucleus	—	—	—	—	—	+	+	+	+
Efferents from ventral thalamic nucleus	—	—	—	—	—	+	+	+	+
Pyramid to spinal cord	—	—	—	—	—	—	+	+	+
Anterior commissure:									
anterior limb	—	—	—	—	—	—	+	+	+
posterior limb	—	—	—	—	—	—	—	tr.	+
Pyriform cortex	—	—	—	—	—	—	—	tr.	+
Corpus callosum	—	—	—	—	—	—	—	tr.	+

nerve. Together these pontine and medullary reticulo-spinal fibres run caudally just ventral to the medial longitudinal fasciculus forming an ill-defined group which comes to lie dorso-medial to the homolateral vestibulo-spinal tract in the ventral funiculus of the spinal cord. Here it is joined by a few fibres from the lateral reticular formation of the medulla which course ventrally through the lateral funiculus of the spinal cord. In foetuses of 78 days (Fig. 1 A) and older these are obscured by a greater number of fibres which enter the lateral funiculus of the spinal cord from the lower medulla and, lying with the rubro-spinal tract, constitute the lateral reticulo-spinal tract. In the 63-day-old foetus a few fibres enter the ventral funiculus of the spinal cord from the ventral reticular formation of the medulla; whether these represent the ventral reticulo-spinal tract or not was not determined.

66-*day foetus*

In this foetus the dorsal spinal roots in the cervical region contain a little myelin, there is some in all parts of the sensory root of the trigeminal nerve, especially the mesencephalic component which can be traced to the level of the inferior colliculus, and in the glosso-pharyngeal, vagus and accessory nerves.

The lateral funiculus of the cervical and upper thoracic spinal cord contains a few medullated fibres of the dorsal spino-cerebellar tract whose fibres pass to the vermis of the cerebellum (Fig. 2 A). A few fibres of the uncinate fasci-

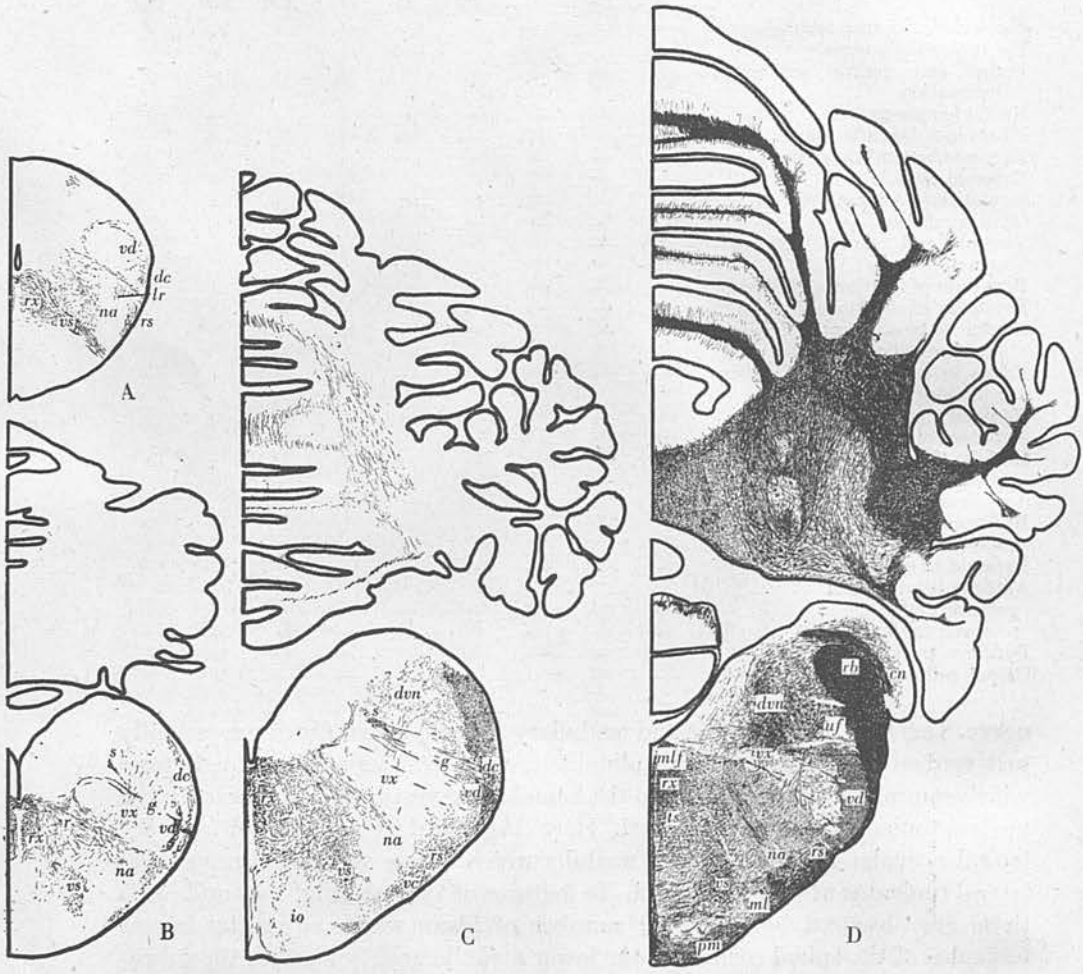

Fig. 1. Drawings of Weigert-Pal preparations of the medulla and cerebellum of three sheep foetuses. A and B, 78-day foetus, ×7·5; C, 96-day foetus, ×7·5; D, 140-day foetus, ×5.

culus arch ventrally from the region of the fastigial nucleus to the vestibular nuclei which also send fibres into the roof nuclei of the cerebellum. The medial longitudinal fasciculus is slightly medullated, especially in its pontine and medullary extent.

78-day foetus

In younger foetuses medullation is confined to the peripheral nerves and central connexions of the vestibular apparatus and the reticulo-spinal pathways. In the 78-day foetus there is a moderate amount of myelin in the

tegmentum of the midbrain (Fig. 5 A). A few fibres of the rubro-spinal tract are medullated and can be traced to the cervical spinal cord. In addition, reticulo-spinal fibres arise from the isthmic region and decussate among a very few fibres of the brachium conjunctivum which cannot be traced forwards to the red nucleus, though the medial longitudinal fasciculus is medullated to the level of the posterior commissure in which are one or two fibres with a myelin sheath.

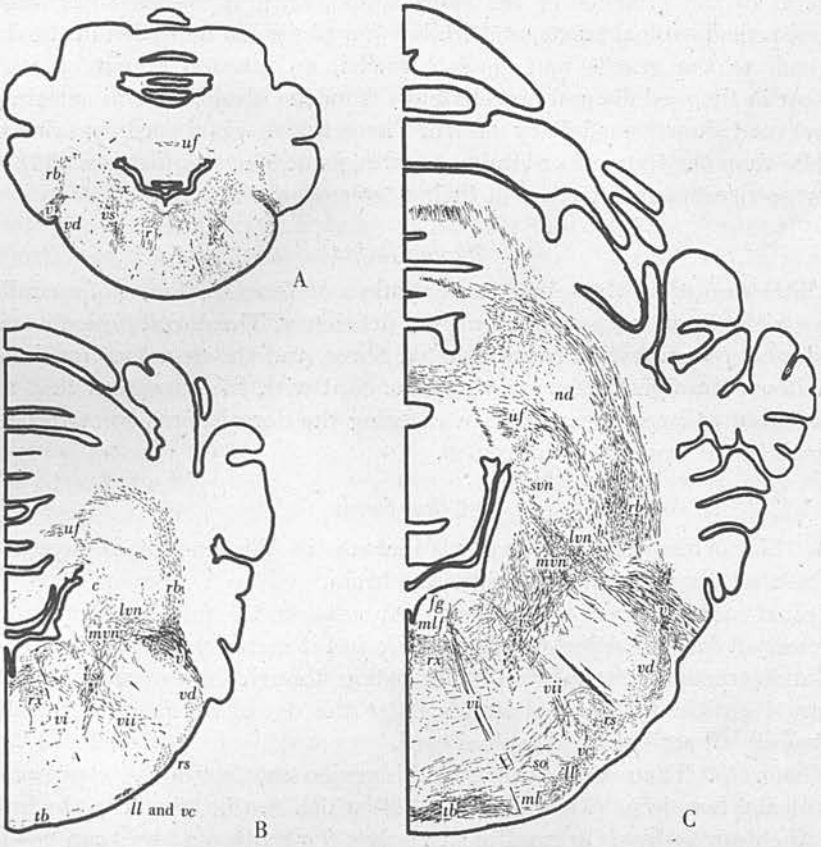

Fig. 2. Drawings of sections through the vestibular nerve in sheep foetuses. Stain Weigert-Pal. A, 66-day foetus, ×6; B, 78-day foetus, ×7·5; C, 96-day foetus, ×7·5.

All the connexions of the vestibular apparatus are more heavily medullated (Fig. 2 B) and include the cerebellar and descending roots. A few crossed vestibulo-spinal fibres are found arising from the nucleus of the latter (Fig. 1 B), and some crossed vestibulo-mesencephalic fibres from the medial nucleus have a myelin sheath. Both cochlear nuclei contain a few medullated fibres, and these can be traced in the striae acousticae and trapezoid body to a finely medullated lateral lemniscus which reaches the inferior colliculus.

tract (Fig. 1 B) contains some myelin, and the number of fibres from pons and medulla is greatly increased (Figs. 1 B

ellum the flocculus contains some medullated fibres, and the erebellar tract can be traced over the brachium conjunctivum llum (Fig. 4 A).

funiculus of the spinal cord contains much more myelin on e presence of the rubro-spinal, both spino-cerebellar and the -spinal tracts, and while a few fibres can be traced in the dorsal e gracile and cuneate nuclei, no internal arcuate fibres are medulla and no collaterals from the dorsal funiculi entering the ow a medullary sheath. The cervical spinal cord contains more te thoracic or lumbo-sacral regions. The two foetuses of 78 days cant difference in their degree of medullation.

84-*day foetus*

uli of the spinal cord contain a uniform scattering of medullated evidence of any localized deficiency. The dorsal funiculi contain centration of medullated fibres, and the degree of myelination oughout the length of the cord with the exception that a few llaterals can be seen entering the dorsal horns from the dorsal upper cervical region.

96-*day foetus*

us there is a marked increase in the amount of myelin and res are present in the forebrain.

There is a considerable increase in the number of medullated niculi especially the dorsal, and it seems certain that the spino- s are medullated by this stage though they were never seen as ies. Many collaterals enter the dorsal horns from the dorsal regions of the spinal cord.

The main advance in this region consists in the appearance of mniscus (Figs. 1 C, 2 C, 4 B) which can be traced to the level of ucleus. In front of this only a few scattered fibres can be found, tful whether the lemniscus reaches the thalamus. In addition, d dorsal external arcuate and olivo-cerebellar fibres are medul- gitudinal fascicles in the pontine and medullary reticular forma- more obvious. The medial longitudinal bundle can be traced to e posterior commissure, where its fibres fan out into the reticular ve the substantia nigra.

r cerebellar peduncle (Fig. 4 B) is well medullated and reaches us but cannot be traced for any great distance farther. The nb of the brachium conjunctivum (Fig. 4 B), which arises in the receives fibres from the ipsilateral tegmentum apparently

originating dorsal to the pons where a few med present. The lateral lobe

Midbrain. Fibres of medullated, and in the to be medullated. Its sensory trigeminal nucl thalamus.

The commissures of t lated.

Lateral to the substar which seems to be form lemniscus and partly fr These can be traced for and also give rise to Me the findings of Magoun

Forebrain. The olfact finely medullated fibres body and pretectal nucl the posterior part of the followed to the cortex contain medullated fibr

The lateral olfactory are not found in the py olfacto-septal fibres pas scattering of medullate no myelin is present in

The habenular and te few medullated fibres a

A small number of fil part of the frontal corte a few are present in the caudally as far as the are a few scattered fib bundle forming the fasc region. In the subthala can be traced to the H_2

This stage is not ass medullated tracts, but of those already seen.

The brachia pontis a a considerable quantity

originating dorsal to the substantia nigra, and can be traced caudally to the pons where a few medullated fibres of the middle cerebellar peduncle are present. The lateral lobes of the cerebellum contain very little myelin.

Midbrain. Fibres of the tecto-spinal and spino-tectal tracts (Fig. 4 B) are medullated, and in the isthmus the central tegmental fasciculus is beginning to be medullated. Its fibres appear to originate mainly from the principal sensory trigeminal nucleus of the same side but could not be traced to the thalamus.

The commissures of the lateral lemniscus and inferior colliculus are medullated.

Lateral to the substantia nigra there is a scattered group of fibres (Fig. 5 B) which seems to be formed partly from the upward continuation of the medial lemniscus and partly from the stratum profundum of the superior colliculus. These can be traced forwards into the lateral part of the fasciculus thalamicus and also give rise to Meynert's commissure. This appearance is in accord with the findings of Magoun & Ranson (1942).

Forebrain. The olfactory and optic tracts contain a considerable number of finely medullated fibres. Optic fibres can be traced to the lateral geniculate body and pretectal nucleus. From the former a few medullated fibres pass into the posterior part of the internal capsule but are so scanty that they cannot be followed to the cortex. Both ventral and dorsal supraoptic commissures contain medullated fibres.

The lateral olfactory tract is medullated over its whole extent, but fibres are not found in the pyriform cortex. From the medial olfactory area a few olfacto-septal fibres pass to the nuclei of the septum pellucidum, and a fine scattering of medullated fibres can be traced to this region in the fimbria but no myelin is present in the anterior column of the fornix.

The habenular and terminal striae both contain a little myelin, and a very few medullated fibres are discernible in the habenulo-peduncular tract.

A small number of fibres with fine medullary sheaths arises from the dorsal part of the frontal cortex. These are almost confined to the sigmoid gyrus but a few are present in the coronal gyrus; they enter the internal capsule and pass caudally as far as the putamen of the lentiform nucleus. In the latter there are a few scattered fibres, and from the globus pallidus a finely medullated bundle forming the fasciculus and ansa lenticularis passes to the subthalamic region. In the subthalamic nucleus there are several myelinated fibres which can be traced to the H_2 Field of Forel.

111-*day foetus*

This stage is not associated with such a great increase in the number of medullated tracts, but rather there is an increase in the degree of medullation of those already seen.

The brachia pontis and all parts of the cerebellar hemispheres now contain a considerable quantity of myelin (Fig. 3 A). In the midbrain (Fig. 5 C) the

brachia of both colliculi are medullated and a few scattered fibres are found in the stratum superficiale of the superior colliculus, with many fibres entering the central grey matter and sweeping ventrally through it towards the oculomotor nucleus and dorsal tegmental decussation. The habenulo-peduncular tract can be followed to the interpeduncular nucleus (Figs. 4 C, 5 C).

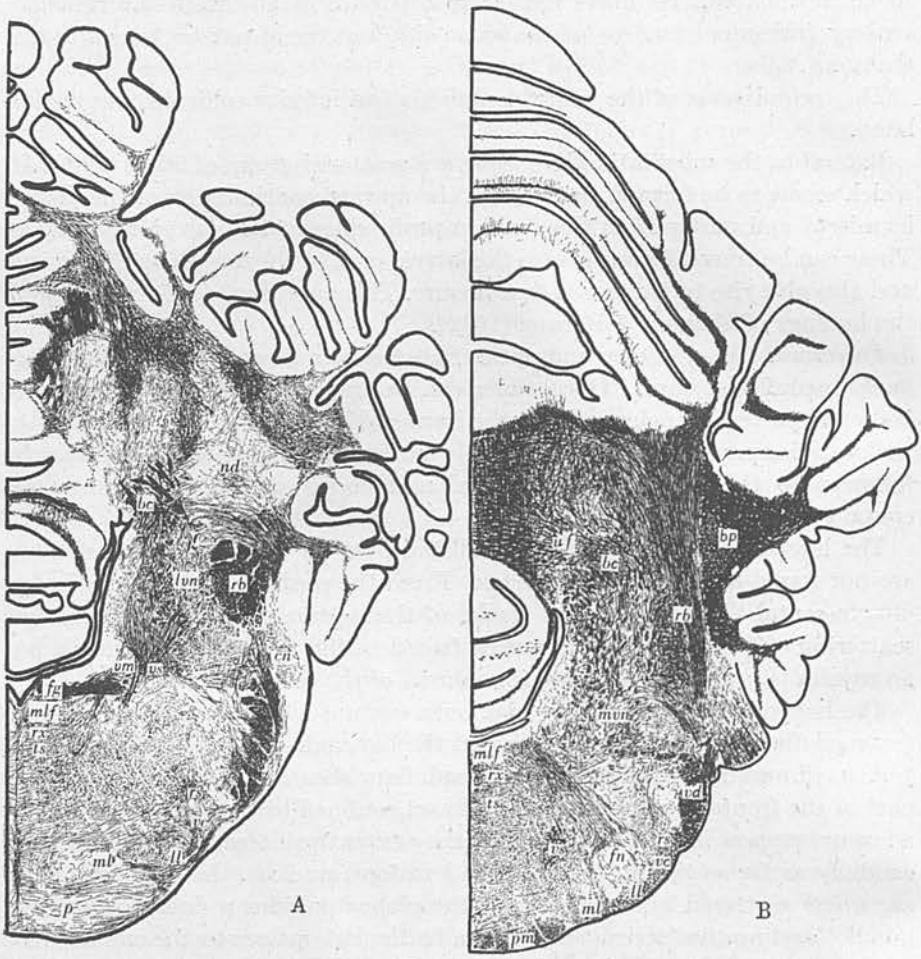

Fig. 3. Drawings of sections through the vestibular nerve in sheep foetuses. Stain Weigert-Pal. A, 111-day foetus, ×6; B, 140-day foetus, ×5.

The mamillary peduncle, the fornix and the mamillo-thalamic tract can all be traced to the mamillary body though the fornix is very poorly medullated.

Fibres of the brachia conjunctiva can now be traced upwards through the red nucleus into the subthalamic region, and both medial lemniscus and spinothalamic fasciculi extend to the thalamus.

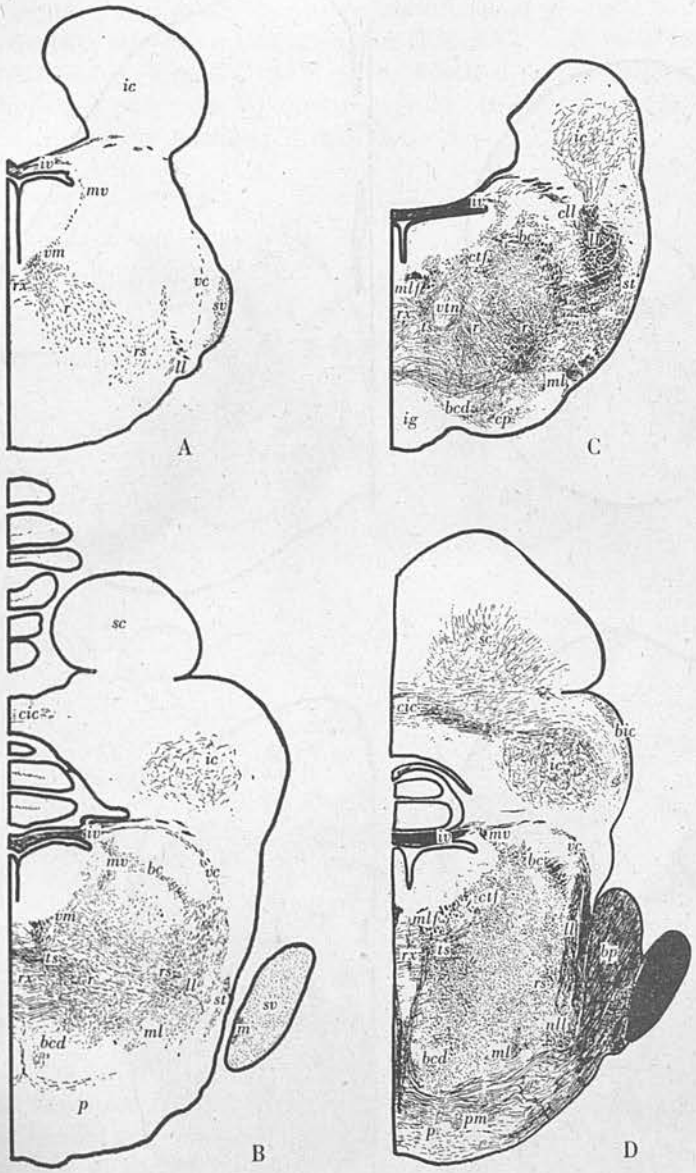

Fig. 4. Drawings of sections through the decussation of the trochlear nerve in sheep foetuses. Stain Weigert-Pal. A, 78-day foetus, ×7·5; B, 96-day foetus, ×7·5; C, 111-day foetus, ×6; D, 140-day foetus, ×5.

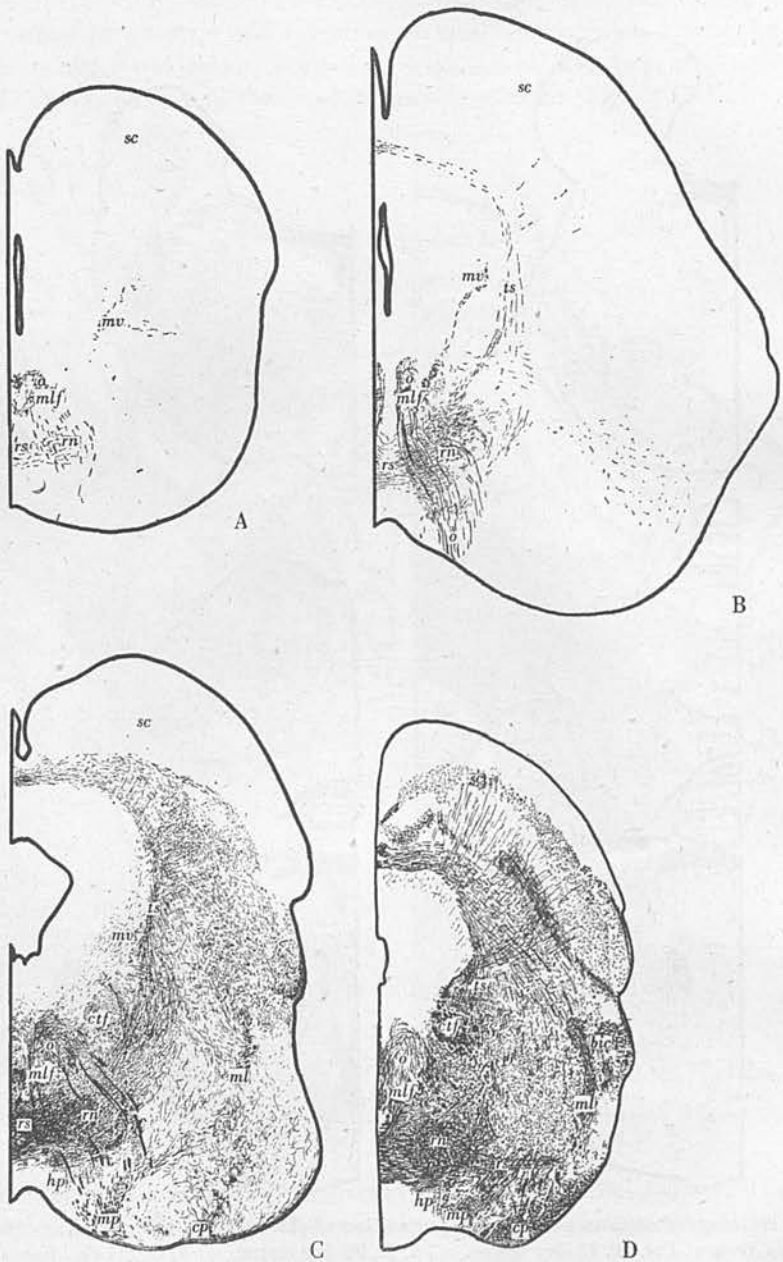

Fig. 5. Drawings of sections through the oculomotor nucleus in sheep foetuses. A, 78-day foetus, ×7·5; B, 96-day foetus, ×7·5; C, 111-day foetus, ×6; D, 140-day foetus, ×5.

Most of the thalamic nuclei, with the exception of the pulvinar and dorsal part of the medial nuclear group, contain a few scattered fibres, the anterior nuclei receiving the mamillo-thalamic fasciculus and giving a well-marked group of efferents into the internal capsule (Fig. 6 C), and while the central tegmental fasciculus (Figs. 4 C, 5 C) can be followed to the nucleus reuniens its cephalic part is poorly medullated, though at 140 days (Fig. 5 D, 7 A) this portion is more heavily medullated than the rest.

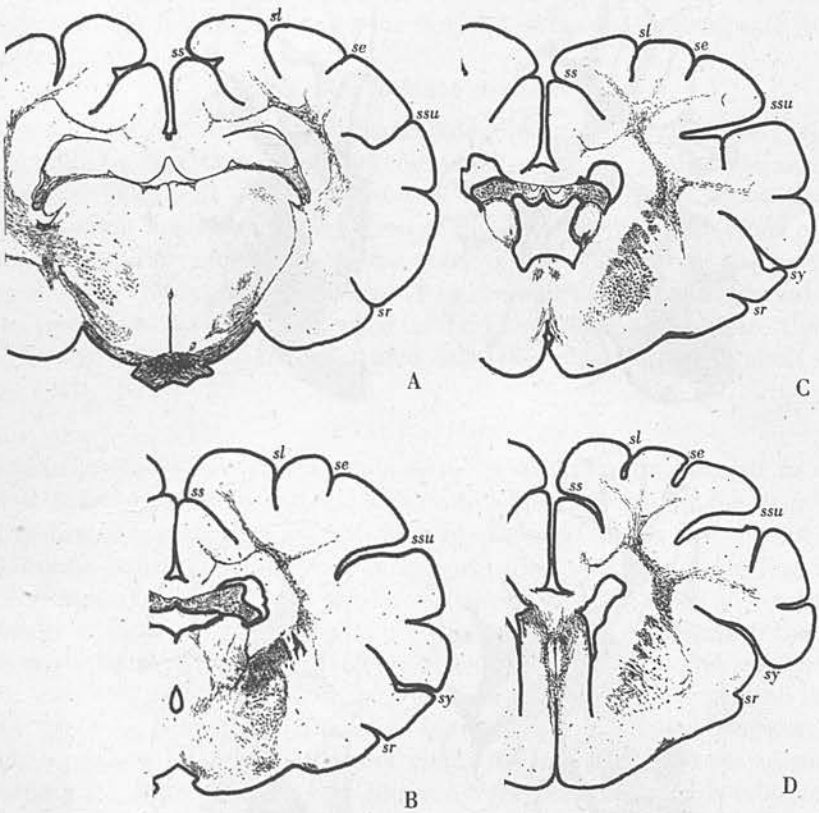

Fig. 6. Drawings of sections through four levels of the forebrain from a sheep foetus of 111 days. Stain Weigert-Pal, ×2.

Efferent fibres are present from the ventral, lateral, anterior, lateral geniculate and medial geniculate nuclei of the thalamus.

The number of medullated cortical connexions is increased though the fibres are not numerous; they are present in the cingulate, coronal, lateral, mid-suprasylvian, posterior suprasylvian, anterior and posterior ectosylvian gyri (Fig. 6).

Efferent cortical fibres can be traced through the corpus striatum into the cerebral peduncle. The great majority of these contain no myelin caudal to

the subthalamic nucleus, but a few (Figs. 5 C, 6 C), whose nature is not determined, pass caudally to the level of the isthmus and appear to end in the ventral part of the tegmentum of the midbrain. There is no myelin in any of the pontine pyramidal fasciculi.

There are no medullated associational fibres in the cortex, and the corpus callosum is devoid of myelin (Fig. 6).

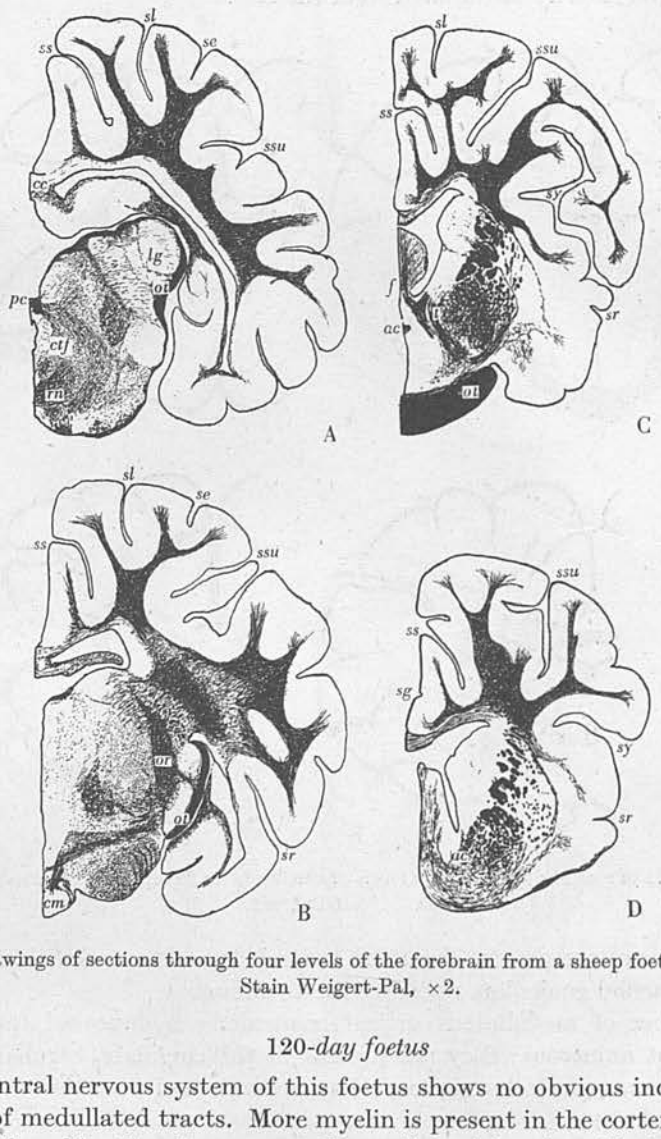

Fig. 7. Drawings of sections through four levels of the forebrain from a sheep foetus of 140 days. Stain Weigert-Pal, ×2.

120-*day foetus*

The central nervous system of this foetus shows no obvious increase in the number of medullated tracts. More myelin is present in the cortex, and a few fibres are found in all regions except the pyriform lobe, the orbital gyri and the extremity of the occipital pole.

Scantily medullated pyramidal fibres can be traced through the pons and a few extend as far caudally as the cervical spinal cord.

All thalamic peduncles contain more myelin and intrathalamic fascicles are more obvious than in the previous foetus, but there is still little myelin in the dorsal part of the medial nuclear group and pulvinar. A few short associational fibres are present in the cortex, but the corpus callosum is devoid of myelin.

The anterior limb of the anterior commissure contains medullated fibres, and though no commissural fibres with a myelin sheath are present in the posterior limb a few derived from the fascicles of the internal capsule run in its lateral part.

130- *and* 140-*day foetuses*

In both of these foetuses all the thalamic connexions are medullated (Fig. 7), and in addition there are medullated fibres in the posterior limb of the anterior commissure (Fig. 7 C) and in the corpus callosum. All parts of the cortex contain several medullated fibres though these are still not numerous in the orbital gyri, the pyriform lobe or the occipital pole; in all regions there are fibres showing the beaded appearance characteristic of early medullation.

The pyramidal tract is much more strongly medullated in the 140- than in the 130-day foetus and can be traced into the upper cervical spinal cord (Figs. 1–3).

DISCUSSION

The present observations on a single series of sheep foetuses cannot be considered exhaustive as there may be considerable variation in the degree of medullation at a given age, not only between different breeds of sheep but also in the same breed. Such a variation is present in the literature dealing with the development of myelin in the human foetus and infant. Table 3 shows the range of variation in the results given in two papers dealing with the first appearance of myelin in man (Lucas Keene & Hewer, 1931; Langworthy, 1933). Some of these variations are very marked involving tracts at all levels of the nervous system, and, though it is not quite clear from Langworthy's (1933) paper how he arrived at a table giving the medullation at ten ages after describing five different ages, it is obvious from his text that his table refers to the first appearance of myelin.

Though the above variations may be the result of differences in material, technical procedures or the level at which some of the tracts are studied, they are too great to allow any conclusions to be drawn about medullation in one foetus and functional development in another.

Despite these differences in man and the fact that the sheep foetuses used in this study have been collected in a random fashion over a period of years, each shows a distinct increase in the degree of medullation over the previous member of the series. Also the two sheep foetuses (268 and 340), 78 days old, show an exactly similar degree of medullation though born in different years. Thus this limited series of observations can be taken to give only a general

midbrain is severed from the pons at this developmental stage, the pontine and medullary segments of the brain demonstrate no evidence of inhibition though they alone contain myelin. Thus the development of myelin is not responsible for the inhibition, the operation merely causing the foetus to revert to a less advanced stage in its functional development without showing any evidence that the presence of myelin in regions caudal to the level of section has altered their functional capabilities. Even when the spinal cord is severed in the cervical region at a time when it contains a moderate number of medullated fibres (70 days), the activity of the trunk and limbs caudal to the section reverts to that found in normal foetuses 36–40 days old when no myelin is present.

There is therefore no evidence that the onset of medullation produces any marked change in the physiological response of the foetus, and the appearance of sustained movements (Barcroft & Barron, 1942) precedes the development of myelin. In contrast to Langworthy's (1926) findings but in agreement with those of Windle (1929), decerebrate rigidity appears in development at a time when the midbrain contains practically no myelin and the rubro-spinal apparatus is quite devoid of it.

These facts are in favour of the view that function precedes medullation and is independent of it for a considerable time. On the other hand, the optic nerve in the sheep begins to be medullated while the foetus has still 50 days to remain *in utero*. Here medullation can be conditioned neither by the onset of normal function in this nervous pathway nor by association with the ability to open the eyes (Langworthy, 1933), for the eyelids are still tightly fused 15 days later and only begin to separate 24 days after medullation in the optic nerve has begun. It may be that the development of myelin in the optic system is initiated by factors quite different from those found elsewhere in the nervous system, but it is clear that the transmission of impulses resulting from light reaching the retina is not a factor in the sheep or in man where the optic nerve also begins to medullate before birth (Lucas Keene & Hewer, 1931; Langworthy, 1933).

The conclusion seems warranted that medullation can occur in the absence of normal function and, though this does not help to clarify the mechanism, that the developing nervous system prepares itself for the demands which are to be put upon it at birth. It is obvious that a sheep is born with a nervous mechanism more highly developed than that of a newborn rabbit, yet each has as little knowledge of the world outside as the other. This inherent development in the absence of function has been demonstrated by Harrison (1904), who has shown that development of functional capabilities in the amphibian nervous system is not retarded by immersion in chloretone which removes from the larva the ability to mould its nervous system by its activity. Similarly, Goodman (1932) states that placing newborn rabbits in the dark for 6 months does not impair medullation of the optic nerve and, though Held (1928) believes that exposure to light hastens this process, it is clear that the absence of function does not prevent or decrease medullation in the optic system.

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f	fornix
fg	facial genu
fn	facial nucleus
g	glossopharyngeal nerve
hp	habenulo-peduncular tract
ic	inferior colliculus
ig	interpeduncular ganglion
io	inferior olive
iv	trochlear nerve
lg	lateral geniculate
ll	lateral lemniscus
lr	lateral reticulo-spinal tract
lvn	lateral vestibular nucleus
m	motor root of trigeminal nerve
ml	medial lemniscus
mlf	medial longitudinal fasciculus
mp	mamillary peduncle
mv	mesencephalic root of trigeminal nerve
mvn	medial vestibular nucleus
na	nucleus ambiguus
nd	nucleus dentatus
nll	nucleus of lateral lemniscus
o	oculomotor nerve or nucleus
or	optic radiation
ot	optic tract
p	pons
pc	posterior commissure
pm	pyramid
r	reticular fibres
rb	restiform body
rn	red nucleus
rs	rubro-spinal tract
rx	medial reticulo-spinal tract and decussation
s	solitary tract
sc	superior colliculus
se	sulcus ectolateralis
sg	sulcus cinguli
sl	sulcus lateralis
so	superior olive
sr	sulcus rhinalis
ss	sulcus splenialis
ssu	sulcus suprasylvianus
st	spino-tectal tract
sv	sensory root of trigeminal nerve
svn	superior vestibular nucleus
sy	sulcus sylvianus
t	stria terminalis
tb	trapezoid body
ts	tecto-spinal tract
uf	uncinate fasciculus
v	vestibular nerve
vc	ventral spino-cerebellar tract
vd	descending trigeminal root
vi	abducens nerve
vii	facial nerve
vm	vestibulo-mesencephalic tract
vs	uncrossed vestibulo-spinal tract
vtn	ventral tegmental nucleus
vx	crossed vestibulo-spinal
x	crossing vestibular fibres

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p	pons
pc	posterior commissure
pm	pyramid
r	reticular fibres
rb	restiform body
rn	red nucleus
rs	rubro-spinal tract
rx	medial reticulo-spinal tract and decussation
s	solitary tract
sc	superior colliculus
se	sulcus ectolateralis
sg	sulcus cinguli
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A NEW SILVER METHOD FOR STAINING PARAFFIN SECTIONS OF THE NERVOUS SYSTEM

By G. J. ROMANES (*Beit Memorial Research Fellow*), *Anatomy School, Cambridge*

In this laboratory only Bayer's pre-war Protargol has proved satisfactory for Bodian's (1936) method for staining paraffin sections of the nervous system. In view of the difficulty of obtaining this substance an alternative method was sought, making use of colloidal silver solutions. It was considered essential that the solutions used should be easily prepared, cheap, as standard as possible and uniformly effective in the hands of technicians and research workers alike.

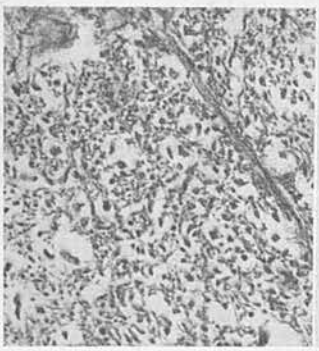

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 1. Section of the lateral column from the spinal cord of a foetal sheep. Fixative, 10% formalin. 4 hr. impregnation. ×312.

Fig. 2. Section of a posterior root ganglion cell from a mouse. Fixative, 10% formalin. 15 hr. impregnation. ×650.

Fig. 3. Section of the corpus striatum from a dog. Fixative, 10% chloral hydrate in 10% formalin. 15 hr. impregnation. ×312.

The solutions are prepared as follows:

1 g. of silver nitrate (old or brown crystals are useless) is dissolved in 20 c.c. of distilled water and just sufficient dilute ammonia is added to dissolve the precipitate formed by the first few drops. This is diluted to 1 l. with distilled water. It forms a stock solution which keeps well and from which the impregnating fluid is prepared. It is important to use dilute ammonia to avoid adding excess.

The impregnating fluid is prepared by adding to 30 c.c. of the above solution 10 c.c. of 1% gelatin, slightly warmed to keep it fluid, 0·8 c.c. of 0·5% tannic acid in distilled water and 0·3 c.c. of pure pyridine, stirring the solution all the time. This solution, which gradually becomes dark brown owing to the formation of colloidal silver, should be made up shortly before use and discarded after impregnation.

METHOD

(1) Paraffin sections of formalin (neutral) fixed nervous tissue, embedded after passing through benzene, are affixed to slides with albumen. It is best to float the sections on alcohol (70%), blot with cigarette paper and dry on a hot plate to ensure satisfactory flattening and firm adherence of the sections to the slides. The sections are brought to water, which need not be distilled, and placed in the impregnating fluid for 4–24 hr. at 58° C.

(2) Rinse rapidly in water.

(3) Develop for 5 min. in:

Pyrogallol	1 g.
Hydroquinone	1 g.
Sodium sulphite cryst.	10 g.
Distilled water	100 c.c.

This developer keeps well in a stoppered bottle and can be used many times.

(4) Wash well in water. Stages 1–4 may be repeated if a more intense impregnation is required.

(5) Transfer to 0·3 % gold chloride in 2 % glacial acetic acid for 5 min.

(6) Rinse in distilled water.

(7) Leave for 10–15 min. in 2 % oxalic acid in 1 % formalin.

(8) Wash well.

(9) Treat with 5 % hypo 5 min.

(10) Wash, dehydrate, clear and cover.

When removed from the staining solution, properly impregnated sections are pale brown in colour and should not darken appreciably in the developer. Under-impregnation leads to intense staining of the background, especially in recently fixed tissue, while over-impregnation produces poor cell staining.

After correct impregnation the sections show purplish-black fibres on a clear background with reddish cells containing well marked neurofibrils. The exact time of impregnation cannot be stated but, in general, telencephalic and diencephalic tissues need longer than the medulla and spinal cord; freshly fixed material requires a more protracted impregnation than that which has been fixed for some time. For the former 10–15 hr. is usually necessary, but the latter may require only 4 hr.

This stain gives poor results with cerebral cortex unless 10 % chloral hydrate is added to the 4 % formaldehyde used for fixation. It also fails to stain boutons in the majority of cases and Purkinje cells of the cerebellum are poorly impregnated, though basket cells and climbing fibres are well stained.

Alcoholic or strongly acid fixatives give poor results, as much neuroglial tissue is stained. With formalin fixation it is not essential for the tissues to be very fresh, and good results have been obtained with tissues fixed 3 days after death. The stain should therefore be useful in studying post-mortem material and works well with rodent, carnivore, ungulate and human tissue.

The mechanism of the stain is not known, but it seems that colloidal silver alone is not active, for no staining results when sufficient tannic acid is added to convert all the ammoniacal silver nitrate to colloidal silver. This may be due to an increase in the size of the colloidal silver particles or may indicate that a trace of ammoniacal silver nitrate is essential. The latter view receives support from the fact that the addition of an ammonium salt to silver proteinates which had previously failed to stain led to some staining of the nerve fibres.

I am indebted to Mr F. Smith of the Chemistry Laboratory, Cambridge, for suggestions and help in preparing the original colloidal silver solutions.

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THE STAINING OF NERVE FIBRES IN PARAFFIN

SECTIONS WITH SILVER

BY

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THE STAINING OF NERVE FIBRES IN PARAFFIN SECTIONS WITH SILVER.

INTRODUCTION.

The primary requirement for all forms of histological research is a series of methods which will consistently demonstrate particular features of a tissue and, wherever possible, indicate the chemical composition of the structure which is stained.

The multitude of methods which exists for staining nervous elements with silver is a good measure of the relatively unsatisfactory nature of all and demonstrates that none fulfils all the criteria necessary for a perfect staining method.

The criteria which were kept in mind during this research were:-

1. That the stain should be specific for nervous elements or, if other structures were stained, they should be differentiated easily by means of colour contrast.
2. That the stain should demonstrate every nervous element (excluding neuroglia) on all occasions with equal certainty, that it should be simple to prepare and independent of any complex substances of unknown chemical constitution.
3. That it should be possible to use such a stain with paraffin sections, thus avoiding the irregular staining and brittle nature of pieces of tissue immersed in a silver solution, and the lack of cohesion between the various parts of frozen sections, while maintaining the advantages of thin serial sections, alternate members of which may be stained by different methods.
4. That the stain should function with tissue from a number of standard fixatives and should not require fixation with a solution which precludes the use of other staining methods.

The/

The earlier silver methods, mainly based on Cajal or Bielschowsky techniques, though excellent for many purposes, all depend on bulk staining or the use of frozen sections and many require very special fixatives. Of these methods few are completely reliable even in the hands of expert technicians and much valuable experimental material may be lost at the last ditch.

More recently a series of techniques for staining paraffin sections has been evolved and some of these are extremely valuable, notably Bodian's (1936) method. All of them have, however, certain drawbacks which are not immediately obvious.

Bodian's method makes use of protargol, a strong silver proteinate, now manufactured only by the Winthrop Chemical Company in the United States. This protargol is the only member of a large series of strong silver proteinates which stains nervous tissue adequately, but nothing is known of its composition beyond the fact that it is a combination of silver with partially hydrolysed egg albumen. Even protargol is not uniformly satisfactory for only certain samples produce the desired result while others (Holmes 1943), even those manufactured by the same Company (Green 1947a), are inactive.

Silver's (1942) method is a modification of one of the standard techniques for silvering glass in the production of mirrors. It makes use of a mixture of Rochelle salt, silver nitrate and potassium sulphide added to an equal volume of ½% protargol; but has the disadvantage that it is complex to prepare, capricious in action and has all the disadvantages of Bodian's technique.

Holmes (1943) gives an excellent review of silver techniques for staining paraffin sections and notes that silver oxide and silver carbonate are more effective for this purpose than silver nitrate. He was apparently unaware of the presence of silver chloride in protargol and gives no indication of having used this salt. Holmes/

Holmes makes use of a very weak solution of silver nitrate (1:10,000 - 1:100,000) with a borax-boric acid buffer. Despite its simplicity this method has not given adequate results in my hands though the reason for this is not at all obvious. In any case it requires variations in pH and silver concentration for different tissues which is unsatisfactory where sections passing through a whole animal or embryo are to be stained.

Pearson and O'Neill (1946) make use of a form of physical development. Sections are first impregnated with silver nitrate and the deposit intensified by means of a solution containing silver nitrate and a reducing substance (hydroquinone) with a protective colloid (gelatin) added to arrest the wholesale deposition of silver. The method is well conceived but leads to the deposition of silver around the axons making them appear thicker than in fact they are, as is the case with many of the block impregnation methods. Also it is not a highly specific stain and for this reason works best with embryonic tissues where few connective tissue fibres are present to complicate the picture. Pearson (1947) has stated that the preliminary use of protargol in addition to the silver nitrate is valuable. Gelatin too is a notoriously variable substance and results may depend on the type used, the presence or absence of traces of mustard oil or sodium chloride perhaps playing an important part in the results obtained.

In a previous publication (Romanes, 1946) a method was described giving results similar to those obtained with Bodian's technique. The solution consists of colloidal silver produced by the action of tannic acid on a weak (0.1%) ammoniacal silver nitrate solution and made alkaline by the additoon of pyridine. This method, though reliable, depends on two variables which are difficult to control. As with all ammoniacal silver nitrate solutions it is difficult to determine the exact end point when all the silver hydroxide/

hydroxide, precipitated by the ammonia, has just been redissolved and, as stated above, variations in the type of gelatin used and the pH of the final solution have a profound result on the impregnation obtained.

The present research was aimed at removing some of these variables.

Since it had been found that Bodian's method gave the best result an attempt was made to analyse the differences between protargol and the other inactive silver proteinates, on the basis of information available in the chemical literature. The significant points may be summarised briefly:

1. Protargol is paler in colour than any other silver proteinate, with the possible exception of freshly prepared silver gelatose (Martindale 1941). This suggests that the particle size of the silver in protargol is smaller than that in other silver proteinates.

2. Protargol alone gives the tryptophane reaction. (Mannich and Gollasch 1927).

3. Protargol while containing about the same total amount of silver (7.91%) as the other proteinates has a percentage (0.83%) of silver as chloride. (Kolthoff and Tomicek, 1925).

From these facts it seems possible that the success of protargol may be due either to the small size of the silver particles, or to the nature of the protein hydrolysate used in its manufacture, or to the presence of cilver chloride.

Tests made of the pH of 1% solutions of various silver proteinates including protargol show that the majority have a pH of about 7.8 - 8. Thus the pH alone cannot be responsible for the failure of some solutions and the success of others.

RESEARCH.

The first attempts were directed to the production of a silver proteinate with characters similar to that of protargol.

Various hydrolysates of albumen, casein and gelatin were prepared by the action of either trypsin or papain or alkalis and the results of hydrolysis were either precipitated by silver nitrate or had small quantities of silver nitrate added to them.

In the former the precipitate was washed and brought into solution by the addition of sufficient alkali to raise the pH to 7.6 - 7.8. In the case of the casein hydrolysates the precipitate was found to redissolve following repeated washing with distilled water. The pH was then between 7.5 - 7.8. Though a small amount dissolved in each wash it was only when this point was reached that the greater part passed into solution.

These solutions ($\pm$ metallic copper) were used in lieu of protargol for impregnating sections, which were subsequently developed and gold toned by the method previously indicated (Romanes,1946). Many of the possible variables were checked by altering the concentration, the pH, the type of alkali added, the fixation of the tissue and the time and temperature of the impregnation.

Despite occasional successes the results were poor and difficult to control, which is not surprising in view of the complicated nature of the solutions used. Certain points,however,did emerge:-

1. The results obtained when the solution was made alkaline either with ammonia, ethylamine or pyridine, were definitely superior to those obtained with sodium or potassium hydroxide.

2. Casein or egg albumen hydrolysates gave good results which could not be obtained with gelatin.

3. The pH range for successful results lay above 6.8 and below 8 with the optimum about 7.8. Below 6.8 - 7 only a greenish, granular staining/

staining of all the tissues resulted, while above 8 all specificity was lost.

4. Impregnation in the cold seldom gave good results while at 58°C in the dark much better impregnation was obtained.

5. Successfully impregnated sections were light brown in colour before development and darkened only slightly in the developer suggesting, as Holmes (1943) found, that the important part of the process consists of a reduction of silver by the tissue itself (vide infra).

Finally to test the value of silver chloride small quantities of sodium chloride, commensurate with the amount found in protargol, were added to the silver precipitate of casein hydrolysate. This addition was found to raise the pH and bring the precipitate partially into solution. This procedure seemed to improve the staining but dissolved the albumen fixing the sections to the slides so that few satisfactory results could be obtained.

In view of the failure with these tests it was decided to investigate the results with silver chloride.

Initially a silver chloride suspension was produced by adding 30 cc of 0.1% silver nitrate to 100 cc of 0.1% gelatin followed by an equivalent amount of 0.1% sodium chloride. This was brought to pH 7.8 with a small quantity of very weak ammonia solution and sections were stained for varying periods at 58°C in the dark. The subsequent phases of developing and gold toning were carried out as before except that 2% oxalic acid without formalin was used to reduce the gold chloride. It was found, as expected, that during impregnation the pH gradually fell, presumably on account of the evaporation of the ammonia, and overnight was reduced to about 7. Much of the silver chloride precipitated out in the first two hours of impregnation, but despite this the stain was good and most of the precipitate on the slides was dissolved by the sodium sulphite in the developer.

In order to maintain the silver chloride in suspension the amount of gelatin was increased but this was found to impair the staining more or less in proportion to the increase in the amount of gelatin added.

It was therefore decided to reduce the concentration of silver chloride in the solution and dispense with the gelatin which was an unsatisfactory constituent. As a result of this it was found that stable silver chloride suspensions in distilled water were obtained with solutions containing not more than 0.01% of silver nitrate and the equivalent amount of sodium chloride. Solutions of this type gave results superior to those obtained with the addition of gelatin.

The next process was to vary each constituent of this simple solution in turn and decide the optimum solution for staining purposes, then to try the effect of adding other substances to the stain and the use of other silver salts.

The concentration of silver nitrate was gradually reduced by a series of steps to 0.0003% with the equivalent quantity of sodium chloride added in each case. This produced very little change and, though it was obvious that the staining at the lowest concentration was slightly less intense than that at the highest concentration, it was practically impossible to differentiate between sections stained in solutions containing 0.003% and 0.0003% of silver nitrate respectively. A standard solution of .003% silver nitrate was therefore chosen for further tests.

VARIATIONS OF pH, SODIUM CHLORIDE CONCENTRATION, TIME OF IMPREGNATION AND TYPE OF FIXATIVE.

As has been pointed out above the pH falls steadily but slowly during impregnation at 58°C. When this fall in pH was allowed to proceed to about 6.5 - 6.9 it was found that the final sections had a greenish colour and showed only a granular, non specific deposition of silver. The actual point at which this change occurred varied with the fixative. Sections of material fixed in formalin showed no loss of specificity/

specificity even if the pH dropped to 6.8, but if chloral hydrate (10%) was added to the formalin this greenish, granular appearance became obvious if the pH dropped below 7.

A similar result was obtained with Bouin fixed material and where metallic copper (vide infra) was added to the impregnating fluid. With these fixatives and with copper it was therefore found necessary to begin impregnation at a higher pH (8) when a standard time of 16 hrs. was given for impregnation.

It is interesting in this connection to note that in the previous publication (Romanes, 1946) it was observed that overimpregnation led to a gradual diminution in the staining of the tissue and, though not indicated at that time, finally to a greenish granular deposit. Recent tests of the pH of the solution then used at different times during impregnation show that overimpregnation is associated with a reduction of the pH below 7.

At first pH readings were made with a glass electrode but it was found that sufficient accuracy could be obtained with comparator papers (Messrs. Johnston & Coy.). As this was a distinct asset for most histological laboratories, all figures given here have been estimated by this means.

The question arose as to whether exactly equivalent amounts of sodium chloride and silver nitrate were essential or not. To test this the concentration of sodium chloride was varied within wide limits. It was found that as the concentration of sodium chloride was reduced below the equivalent amount the stain became denser and progressivel less specific giving results similar to those obtained when silver nitrate (0.003%) was used alone. On the other hand when the sodium chloride exceeded the equivalent amount no specific staining could be obtained and the results were similar to those which followed when the pH of the standard solution was allowed to fall too low. In order to avoid this difficulty it was decided to keep the silver nitrate solution very/

very slightly higher (3:1) than the equivalent amount (2.9:1) with slight loss of specificity but avoiding the above result which could easily supervene from slight errors in measurement.

An attempt was made to standardise the impregnation time. It soon became obvious that it was not in a simple inverse relation to the temperature but varied with the type of tissue used and to a less extent with the nature of the fixative. It was also noted that, provided the pH was not allowed to fall too low, long periods of impregnation produced no appreciable difference. For this reason and because those tissues which required the longest impregnation (cerebral cortex) had reached their maximun degree of staining in less than 16 hrs. at 58°C this was taken as the standard time.

OTHER SILVER SALTS.

At this stage it became necessary to determine whether or not silver chloride was necessary for the success of this staining method, for, if it was, it might explain why certain batches of gelatin (some of which contain sodium chloride) proved successful in the previous stain (Romanes, 1946) while others did not.

To determine this, certain other silver salts were used in the same way, being made alkaline with ammonia:

1. Silver nitrate. When this salt was used alone even in high dilution the staining was diffuse and nonspecific for nervous tissue, despite considerable variation in the pH range.
2. Silver bromide and iodide. These two halides were both tested extensively because of their similarity in physical properties to silver chloride. All three are light sensitive but both the bromide and the iodide are much less soluble in water and in ammonium hydroxide than the chloride.

The salts were prepared by adding equivalent amounts of sodium bromide or sodium iodide to an 0.003% solution of silver nitrate. The pH was then regulated with weak ammonia and sections immersed in the solutions for 16 hrs at 58°C. In no case was any staining obtained. This may have been due either to the low solubilities in water and ammonium hydroxide or to some other property of these salts. The latter seems not unlikely for the solubility of silver bromide at 100°C (.0.00037 gm / 100 cc) is considerably greater than the solubility of silver chloride at 10°C (0.000089 gm / 100 cc) and both the bromide and iodide are slightly soluble in ammonia.

Many other silver salts were prepared by the addition of equivalent amounts of the appropriate sodium salt to 0.003% silver nitrate. These can be classified into two main groups:

1. Silver salts insoluble at this dilution. These included chromate, ferricyanide, and thiocyanate. In all cases no stain was obtained, which was probably accounted for by the stability of these salts.

2. Silver salts soluble at this dilution. These included phosphate, carbonate, citrate, fluoride, oxylate and tungstate. They, as was expected, gave results which were, in the main, indistinguishable from those obtained with silver nitrate.

VARIATIONS IN ALKALI.

Ammonia was used in all the previous experiments principally because of its close association with silver techniques and because it was a solvent for silver chloride.

In a series of experiments it was replaced by other alkalis namely, pyridine, ethylamine and sodium hydroxide.

With both pyridine and ethylamine results were obtained which were similar to those where ammonia had been used, except that the final result was redder and the nerve fibres did not contrast so well with the background/

background. With sodium hydroxide, which does not dissolve silver chloride, some positive results were obtained. A few of these were almost as good as with ammonia but it was obvious that it was difficult to control, small differences in pH producing a profound effect which was not obtained with ammonia. It was thought at first that the pH would remain more constant with sodium hydroxide but was found to fall in 16 hrs. at 58°C. from 7.6 to 6.5. Despite this and contrary to the findings with ammonia, the results were not granular or greenish in colour.

Because of the difficulty in controlling the results with sodium hydroxide and the final colour of the sections with pyridine and ethylamine, these alkalis were discarded in favour of ammonium hydroxide.

ATTEMPTS AT INCREASING THE SENSITIVITY OF THE STAIN.

Certain features of the silver chloride-ammonia stain which were discovered cast some light on the mechanism of its action.

1. The sections are light brown in colour when removed from the impregnating solution. This suggests, as was found by Holmes (1943), that the silver was reduced by the tissue, and was confirmed by omitting the developing bath, washing the sections and gold toning. Despite the the absence of development the sections showed a considerable degree of staining which was only slightly less than that obtained when development was practised.

Double Impregnation.

A further test was to impregnate sections in two or more baths of the standard stain each for 16 hrs. at 58°C. without intermediate development. This process failed to produce any marked increase in the intensity of staining suggesting that the silver acceptors, presumably reducing agents, in the tissue had been saturated at the first impregnation. This was in marked contrast to Silver's (1942) findings which led him to suggest that negatively charged silver micelles were deposited on positively charged proteins and that the process was a continuous one and could be carried on till large/

large quantities of silver had been deposited. Just how this was achieved is not clear but the fact that the present findings agreed with Holmes (1943) suggest that these methods work on a different principle. Tests carried out with physical developers of different types prior to gold toning showed that the initial impregnation could be intensified in this manner. The deposition of extra silver produced a granular effect on the surface of the fibres increasing their apparent thickness. This was in marked contrast to the microscopically agranular deposit which resulted from the standard method.

2. In no case had it been possible, either by altering the fixative or the constituents of the stain, to obtain an impregnation of every nervous structure, especially in the cerebral cortex and in the case of olfactory and postganglionic autonomic nerve fibres.

Because of this latter fact and the evidence pointing to the reducing power of the tissue as the principle agent, certain lines of investigation for increasing the sensitivity were attempted.

1. To increase the sensitivity of the silver chloride by light and by the use of traces of mustard oil as in photographic emulsion sensitisation.

   Exposing the solution to light during impregnation destroyed the stain and the addition of mustard oil in minute traces produced no significant result.

2. Three reducing substances were considered likely participants in the reaction:

   (a) Suphidryl groups
   (b) Ascorbic acid
   (c) Aldehydes.

Sections of tissue were treated with potassium cyanide solutions of varying strengths with a view to unmasking sulphidryl groups. At some concentrations of KCN, notably $<1\%$, there was some improvement/

improvement in the stain, but treatment with alkaline solutions of the same pH produced similar results. It was therefore presumed that no unmasked sulphidryl groups were present in the sections or that sulphidryl groups were not taking part in the reaction.

Other sections were treated prior to impregnation with thioglycollic and with a view to breaking S-S linkages and producing sulphidryl groups. This likewise produced no noticable change.

It was therefore considered that SH groups were not responsible for the reaction.

In another series of tests sections were treated with ascorbic acid by immersion in solutions of various strengths for different times. It seemed that in some cases the stain was improved but this was not particularly obvious and no conclusion could be drawn.

It was thought likely, in view of the above tests, that aldehydes were responsible in some measure at least. This view has recently been substantiated by Hsu-Mu Liang (1947) who has stained fresh and fixed nerve fibres with Schiff's reagent.

3. Cajal in some of his methods to demonstrate the finer structures of the nervous system (boutons) used chloral hydrate as a fixative. De Castro in modifications of Cajal's methods made use of both chloral hydrate and urethane in fixing fluids. The significance of these substances is not known but empirical tests were made of the result of adding small quantities (circa 0.1%) of these substances to the impregnating solution.

In the case of chloral hydrate the results were impaired and the silver chloride in suspension was partially reduced. With urethane no significant change was noticed, though Green (1948) has found that the addition of this substance to protargol improves the staining of fine fibres in the cerebral cortex.

COPPER/

COPPER.

Bodian (1936) makes use of the addition of metallic copper to the protargol solutions. This is said to "activate" the protargol. Holmes (1943) has demonstrated that the copper produces a steady fall in the pH of the protargol and presumes that the changing pH allows the solution at some time to reach the optimum for staining different nervous tissues. This might account for the fact, mentioned above, that when metallic copper is added to the silver chloride - ammonia stain a higher initial pH is required to obtain adequate staining. This can be, however, only a partial explanation with the present stain because the addition of metallic copper to the solution, though requiring a higher initial pH for adequate staining, produces a stain whose characteristics are quite different from those of the simple stain.

This is noticable at the end of impregnation when the sections are seen to be stained a reddish brown colour in contrast with the light brown colour normally seen. This reddish-brown colour is principally concentrated in the nervous tissues, bone and muscle, and though other tissues may appear stained after gold toning, especially when the treatment with oxalic acid is prolonged beyond 3 - 5 min, the nerve fibres are everywhere more intensely stained. The colour range which is normally between red nuclei and muscle fibres and purple to black nerve fibres has, with copper, turned to one of light grey muscle fibres, grey nuclei with black nucleoli and black nerve fibres.

It would seem then that the addition of metallic copper to the stain while removing much silver from the solution increases the specificity of the reaction and alters the nature of the substance deposited in the tissue. This occurs even when the pH with copper does not fall below 7.3 at which point control sections in the standard solution without copper show the usual distribution of staining in the tissues with the normal colour range. This seems to indicate that it is not simply the change in pH which is important, but suggests that the/

the copper which passes into solution is in some way concerned with the impregnation.

To test this minute traces of various copper salts were added to the standard staining solutions. In all cases no specific staining could be obtained and a similar result was found when weak solutions of copper hydroxide in ammonia were used in place of the silver solution.

The part played by the copper is not at all clear but it is known that the addition of small quantities of the salts of heavy metals, including copper, to photographic emulsions increases their sensitivity to light by acting as centres of reduction or facilitating the formation of such centres.

FIXATION; DECALCIFICATION, ETC.

The part played by the fixative employed for treating the fresh tissue prior to the use of Bodian's method has been examined by many authors (Bodian 1937. Davenport et al 1938 et seq). With the present stain a wide range of fixatives has been employed and good results have been obtained with formalin (10%), Bouin, Carnoy (without mercuric chloride) and acetic (5%) formol (5%) alcohol (70%). With Bouin fixed material the picric acid is removed by means of several changes of 70% alcohol containing 2% .880 ammonium hydroxide. This step seems to improve the staining properties considerably and has been used with advantage after acetic-formol-alcohol and Carnoy fixation.

For peripheral nerves the best results have been obtained with Carnoy and acetic formol alcohol, while brain stem and spinal cord sections are well stained after formol fixation (especially with 10% chloral hydrate). In the spinal cord boutons have been regularly demonstrated after formol fixation but a strange reciprocal staining has been noticed, some of the cells in a single section of the spinal cord demonstrating neurofibrils clearly but few boutons, while others show few cytoplasmic structures and a large number of boutons. No exclusive anatomical distribution of these two kinds of cell could be determined and it seems likely therefore that there may be a variation in the distribution/

distribution of the silver acceptors in different functional states of the cell or in different types of cell. It is obvious that changes in pH of the stain cannot be responsible for the differences seen here as Silver (1942) has suggested.

With all the fixatives there is a tendency, in sections of the central nervous system, for the axons and dendrites of nerve cells to be stained but only the nuclei of the cell bodies to show up clearly. There are certain interesting exceptions where the cell bodies are stained with considerable regularity. These include all motor neurones, i.e. anterior horn cells and motor cranial nerve nuclei and the giant pyramidal cells of Betz. All root ganglion cells are stained as are the cells of the mesencephalic root of the trigeminal nerve. It would seem that all these cells have certain chemical properties in common containing in moderate amount a silver acceptor which is a reducing agent, probably an aldehyde.

Decalcification has, where necessary, been carried out by means of Bensley's decalcifying fluid (50% formic acid and 20% sodium citrate in equal parts) which was suggested by Green (1947b), who found that the use of this fluid improved the subsequent impregnation. This has been confirmed with the present stain and pieces of tissue were routinely passed through this fluid before dehydration and embedding.

The developing solution used in all these tests was:

Hydroquinone	1 gm
Sodium sulphite cryst	10 gm
Water to	100 ml

This solution has been found valuable in previous tests and though it may be that the high concentration of sodium sulphite dissolves out some unreduced silver the end result is better with this concentration than with lower percentages of sodium sulphite. Whether this is a result of the pH or merely of the sulphite is not known but Holmes (1943) in contrast to Silver (1942) is of the opinion that the pH does not materially affect the stain. The temperature of development is of some significance, for with temperatures below 15^{0}C hydroquinone acts slowly and incompletely.

The/

The final method was as follows:-

1. Pieces of tissue fixed in formalin, bouin, carnoy (without mercuric chloride) acetic-formol-alcohol, or acetic alcohol are treated for a few hours in 2% 0.880 ammonia in 70% alcohol, passed through Bensley's decalcifier for sufficient time to allow penetration, washed thoroughly in water, dehydrated and embedded in paraffin.

2. Paraffin sections mounted on slides with albumen are placed in the following solution for 16 hrs. at 58°C in the dark:-

0.1% silver nitrate	3 ml
Distilled water	95 ml
Mix thoroughly and add 0.1% sodium chloride(analytical)	1 ml

Mix thoroughly and bring to pH 7.8 (Johnston Comparator paper 6883) by adding a few drops of very dilute ammonia. A very little of the silver chloride suspension is dissolved by this but the appearance does not change materially. The solution should be made up just before use and not exposed to bright light for long.

3. Remove the sections and place directly in the following solution for 5 min. at 18-20°C.:-

Hydroquinone	1 gm
Sodium sulphite cryst.	10 gm
Water to	100 ml.

4. Wash well in tap water and pass through distilled water into
5. 0.5% gold chloride for 10 min.
6. Wash sections in distilled water, two changes, for 1 minute.
7. Reduce in oxalic acid 2% for 3-5 minutes. Usually it is not advisable to exceed 5 minutes, for non nervous structures tend to develop up and no marked improvement in the nervous tissue is gained by prolonging the oxalic acid.
8. Wash well in running tap water.
9. 5% hypo 3-5 minutes.
10. Wash well, dehydrate clear and cover.

Results/

Results. Nerve fibres purple to black. Nuclei red. Neurofibrils purple, keratin yellow. Bone cells black.

If it is desired to obtain black nerve fibres throughout, raise the pH of the impregnating fluid to 8.3 and add 5 gm. of metallic copper in the form of narrow guage wire to the solution, otherwise treat as before.

CONCLUSIONS.

The series of experiments described above has brought to light certain characters of a process whereby nerve fibres may be stained. The most important feature is the reduction of a silver salt by some substance, or substances, present in the nerve fibres, the nuclei of all cells, the cytoplasm of certain groups of nerve cells, myofibrils, bone cells and many other situations. The exact nature of the reducing substance is unknown and it is unlikely that any single substance is responsible for the reaction in all the tissues mentioned above. At least there is no evidence to suggest that the stain is chemically specific even though it demonstrates the same structures with complete regularity.

Many silver salts have been used in the tests but only silver chloride has proved satisfactory, which is interesting in view of the presence of this substance in protargol. Why this salt should have produced results where silver iodide and silver bromide failed is not clear.

At the beginning certain criteria for a perfect silver method were set out. Of these the greater number have been fulfilled with one important exception, the failure to stain every nervous element. The outstanding failures were with the olfactory nerves, postganglionic autonomic nerve fibres and certain nerve cells. The two groups of fibres have one feature in common, the absence of a myelin sheath demonstrable by standard techniques. This would seem to suggest that the presence of such a sheath is associated with the ability to reduce silver chloride. The difference goes much deeper than this, for long before any myelin is present it is much more simple to stain the fibres which will develop a myelin sheath than those which will not. Also no difficulty is encountered in staining the fine non medullated parts of medullated nerve fibres (Plates 1 and 2).

It/

It is undeniably true that in the earliest stages of development the staining of nerve fibres is a matter of some difficulty, but this is particularly during the stage of rapid growth of the nerve cell processes. Whatever the reason for this failure may be it indicates that these nerve fibres are in some chemical particular different from the others.

Despite these failures and the inability to intensify the reducing power of the nerve fibres prior to impregnation, the method described above has proved to stain nerve fibres with great regularity while at the same time demonstrating many other elements but in such a way that they can be differentiated clearly from the nerves on account of differences in colour. A fact which cannot easily be demonstrated in monochrome reproductions

The method has proved valuable with a large range of fixatives, is simple to prepare and the solutions are stable and can be made up in bulk ready for use. Only the weak ammonia solution may vary in concentration with the passage of time, but this is of no importance since the pH is tested on each occasion and the addition of a greater or lesser quantity of water with the ammonia makes no difference within quite wide limits.

The silver chloride in the solution would seem to be the active principle in this method rather than that part of it which is dissolved by the ammonia, for when alkalis which do not dissolve silver chloride are used in place of ammonia, results of a similar character have been obtained. It would seem not unlikely in view of this and the fact that the stain is active only when the temperature is raised, that the small quantity of silver chloride in solution is gradually removed by the tissue and continually replaced by the fine suspension of silver chloride. This theory is borne out by the fact that the silver chloride gradually disappears from the solution when a large number of sections are impregnated in a relatively small volume of solution, but remains throughout, apparently unchanged, when only one or two sections are treated in the same volume.

ACKNOWLEDGMENTS/

ACKNOWLEDGMENTS

I am indebted to Professor Marrian and the Staff of the Medical Chemistry Department for much help and many useful suggestions during the course of this work. Also to Dr. M. Ritchie of the Chemistry Department for explaining many of the mysteries of the photochemical reactions of silver.

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ILLUSTRATIONS

All the photomicrographs shown were taken on Ilford microneg film without a filter and are all at the same magnification. The sections which they portray were stained by the standard method without copper, but the contrast is less obvious in the monochrome photographs than the originals.

PLATE 1. All the sections are of mouse tissue fixed in acetic-formol-alcohol and decalcified in Bensley's solution. No counterstain.

Fig.1. Sensory ending in the mucous membrane of the hard palate. Section 10μ.

Fig.2. Section through the organ of Corti showing the canal with nerve fibres crossing it. 15μ section.

Fig.3. Part of a muscle spindle showing the complicated nerve loops, cell nuclei and narrow intrafusal muscle fibres. 10μ section.

Fig.4. Motor nerve endings cut tangentially on the surface of two muscle fibres. 10μ section.

PLATE 2.

Fig.1. Cross section through the vibrissa of a mouse. Note the nerve fibres lying inside the outer sheath beneath the inner sheath. Acetic-formol-alcohol. 10μ section.

Figs.2, 3 & 4. Sections taken from dog material.

Fig.2. A section tangential to a cell of the intermediomedial group in spinal cord showing boutons in knob and ring forms. Formalin 10% with 10% chloral hydrate. 10μ section.

Figs. 3 & 4. Sections of giant pyramidal cells from the motor area of the cerebral cortex. 10% formalin. 10μ sections.

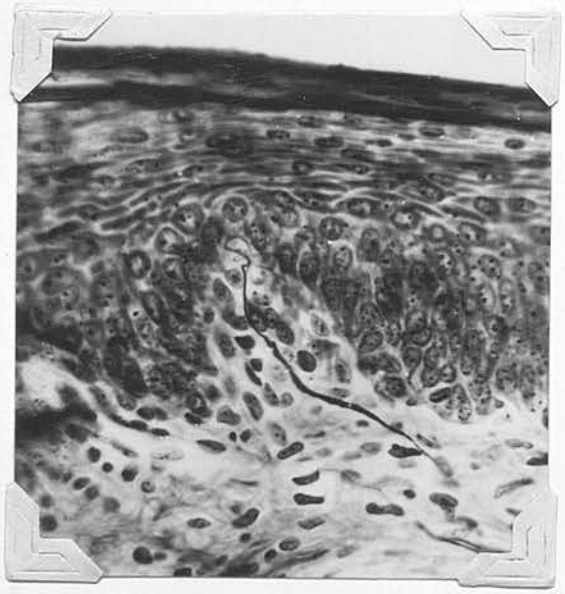

Fig. 1.

Fig. 2.

Fig. 3.

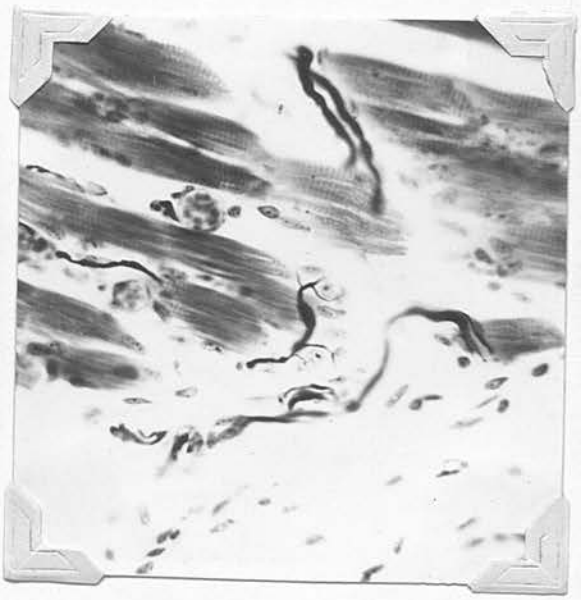

Fig. 4.

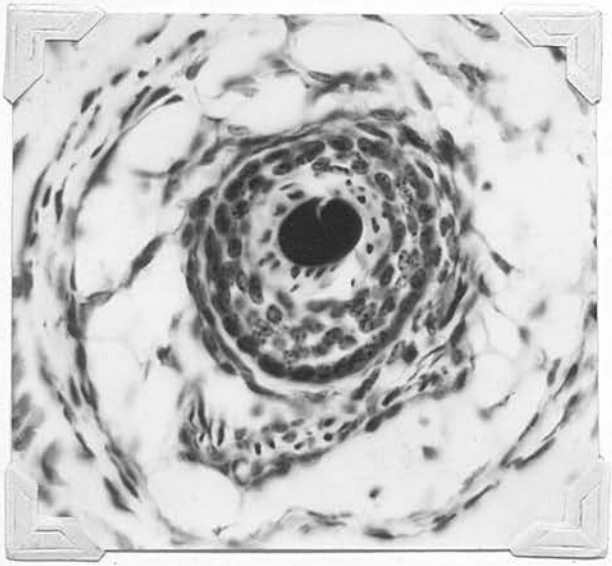

Fig. 1.

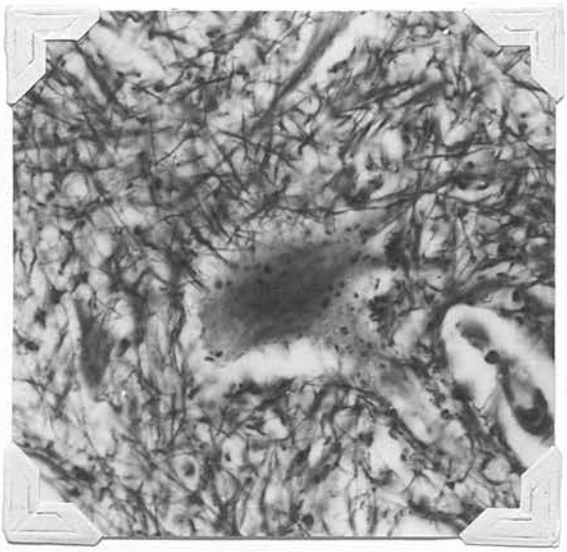

Fig. 2.

Fig. 3.

Fig. 4.